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Mechanistic principles of antisense targets for the treatment of Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy (SMA) is a major neurodegenerative disorder of children and infants. SMA is primarily caused by low levels of SMN protein owing to deletions or mutations of the *survival motor neuron 1 (SMN1)* gene. *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for the loss of the production of the functional SMN protein due to predominant skipping of exon 7. Several compounds, including antisense oligonucleotides (ASOs) that elevate SMN protein from *SMN2* hold the promise for treatment. An ASO-based drug currently under phase 3 clinical trial employs intronic splicing silencer N1 (ISS-N1) as its target. Cumulative studies on the ISS-N1 reveal a wealth of information with significance to the overall therapeutic development for SMA. Here we summarize the mechanistic principles behind various antisense targets currently available for SMA therapy.

Keywords

Antisense oligonucleotide; ASO; SMN; SMA; splicing; ISS-N1; GCRS; ISS-N2; Element 1; Dual-masking

Genetics of spinal muscular atrophy

Spinal muscular atrophy (SMA) is a devastating genetic neurodegenerative disease of children and infants [1–4]. Low levels of Survival Motor Neuron (SMN) protein due to

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deletion or mutation of *SMN1* gene is a primary cause of SMA [5,6]. SMN is a multifunctional protein with distinct functional domains including tudor, nucleic acid binding, self-association, YG-box, proline-rich and calpain cleavage domains (reviewed in [7]). Underscoring the importance of every functional domain, point mutations throughout SMN have been linked to SMA [7]. A nearly identical paralog, *SMN2*, fails to fully compensate for the loss of *SMN1* due to a critical C to T transition at position 6 (C6U transition in transcript) in exon 7 [8]. C6U leads to exon 7 skipping during pre-mRNA splicing of *SMN2*; as a consequence, a truncated highly unstable protein (SMN⁷) is produced [9–12]. *SMN2* can partially compensate the defects caused by the lack of *SMN1* since it (*SMN2*) produces low levels of full-length SMN [5,13]. Confirming the disease-modifying role of *SMN2*, SMA patients with a high *SMN2* copy number manifest reduced SMA severity [14–16]. The disease spectrum of SMA is broad and mouse models representing different severities have been developed [17–21]. Early manifestations captured in the severe mouse models of disease are motor neuron hyperexcitability and morphological and functional defects of peripheral neuromuscular junctions (NMJs) and central synaptic inputs [22–30]. Some of these SMA mouse models also capture accompanying defects in other tissues including heart, intestine, liver, pancreas and lung [30–36]. SMA severity is likely to depend upon several factors that affect SMN levels through transcription, splicing, translation, posttranslational modifications and protein-protein interactions [37–44]. Factors independent of SMN could also influence the severity of SMA and/or cause SMA [45]. Additionally, environmental conditions, particularly hypoxia and oxidative stress may also increase disease severity [46,47].

Therapeutic approaches of SMA

Given the large number of potentially contributing variables (genes and environment) that could impact the severity of SMA, effectiveness of a specific treatment may differ among patients. Various approaches tested for SMA therapy in either mice or human trials include histone deacetylase (HDAC) inhibitors, translation read-through compounds, quinazolines, hydroxyurea, polypeptide- and protein-based therapies, antisense oligonucleotide (ASO)-based therapies, gene-based therapies and stem cell-based therapies [48, reviewed in 49–55]. ASO-based approaches aimed at exon 7 splicing correction have employed several targets including the 3' splice site (3' ss) of exon 8 [56], element 1 (57), intronic splicing silencer N1 (ISS-N1) [58], GC-rich sequence (GCRS) [59], ISTL1 and ISS-N2 [60] and the 100th position of intron 7 (Figure 1) [61]. Among these, the largest number of studies has been done on ISS-N1-targeting ASOs [64], since the first report published in 2006 [58]. ISIS-SMNR_x, an ISS-N1-targeting drug, is currently in a phase 3 clinical trial [65]. If successful, ISIS-SMNR_x will become the first ASO-based drug to elevate the levels of a fully functional protein by stimulating the inclusion of an exon. Selection of the target site is a key to the splicing modulation by an ASO. An ASO that tightly anneals to an exonic target may interfere with nuclear export (of mRNA) and/or block mRNA translation even if that ASO is highly efficient in promoting exon inclusion [64]. Hence, an effective ASO-based drug for SMA would likely be the one that anneals to an intronic target. Several recent reviews provide updates on ASO-based therapies of genetic diseases including SMA [66–68]. There are numerous mechanisms by which an ASO can modulate pre-mRNA splicing. These

include but not limited to the displacement of a transacting factor on pre-mRNA, structural remodeling of pre-mRNA, recruitment of novel transacting factors on pre-mRNA, transcriptional pausing and co-transcriptional splicing regulation. The purpose of this report is to furnish a brief account of mechanisms of ASO-based approaches that are being considered for SMA therapy. The mechanism of *SMN* exon 7 splicing regulation has been reviewed elsewhere [69].

Antisense therapy based on canonical targets

An ASO-based drug for SMA can be developed based on targets of various nature. For the sake of simplification we categorize antisense targets as canonical and non-canonical targets. A canonical target for SMA therapy is a sequence motif that imparts a strong negative effect on *SMN2* exon 7 splicing. As per rule, deletions or mutations of a canonical target should promote *SMN2* exon 7 inclusion. Hence, sequestration of such motif by an ASO should also restore *SMN2* exon 7 inclusion. Among desired features of an ideal canonical target are positioning and accessibility as well as strength and specificity of the ASO:target interactions. Characteristics of an ideal ASO are aqueous solubility, high stability, low toxicity and an efficient means of delivery. Since a number of ASO-based studies have been successfully conducted employing ISS-N1 as a target, we begin by providing the background information on ISS-N1. The discovery of ISS-N1 was a direct consequence of the findings in a previous study of in vivo selection of the entire exon 7 [70]. The purpose of in vivo selection was to uncover the contribution (role) of every single position (residue) of exon 7 in inclusion/exclusion of exon 7 during pre-mRNA splicing of *SMN1/SMN2* in a given cell type. Of note, most early studies focused on strengthening the 3' ss as a possible means to induce *SMN2* exon 7 inclusion, since C6U creates an inhibitory context very close to the 3' ss of exon 7 [71–74]. Surprisingly, the results of in vivo selection revealed that the 5' ss of exon 7 is also very weak. Indeed, a single nucleotide substitution that improved the 5' ss of exon 7 resulted in *SMN2* exon 7 inclusion even in the presence of an abrogated Tra2 β 1 motif that was considered to be an essential element for the inclusion of exon 7 in both *SMN1* and *SMN2* [70]. Of note, a recent in vivo study showed Tra2 β 1 dispensable for inclusion of the *SMN* exon 7 [75], underscoring that the one-motif-one protein hypothesis needs revision as multiple proteins may interact with the same and/or overlapping motifs to modulate pre-mRNA splicing [76]. Findings of in vivo selection triggered the quest for novel regulatory elements that weaken and/or sequester the 5' ss of *SMN2* exon 7. This required a thorough analysis of cis-regulatory elements as well as structural elements that may affect the accessibility of the 5' ss of exon 7. Traditionally, the 5' ss of a human U2-type intron is defined by the last 3 and the first 6 residues of an exon and an intron, respectively [77]. However, the accessibility of the 5' ss could be modulated by RNA-RNA and RNA-proteins interactions involving motifs away from the 5' ss of an exon [78–81].

In the context of *SMN2*, several overlapping deletions downstream of the 5' ss promoted exon 7 inclusion [58]. The shortest of these deletions with the most prominent stimulatory effect on *SMN2* exon 7 splicing was a 15-nt long sequence (CCAGCAUUAUGAAAG) that spanned the 10th through 24th positions of intron 7. This unique 15-nt-long sequence was named ISS-N1 (Figure 1) [58]. Underscoring the inhibitory nature of ISS-N1, its

sequestration by an ASO fully restored *SMN2* exon 7 inclusion in SMA patient cells [58]. This study utilized a 2OMe ASO that is characterized by a phosphorothioate backbone and 2'-O-methyl modifications at every position. Two key observations were significant for the future development of an ASO-based therapy employing ISS-N1 as a target. First, the ISS-N1-targeting ASO produced a robust stimulatory effect on *SMN2* exon 7 splicing in SMA patient cells even at low concentration e.g. 5 nM. Second, the antisense effect against the ISS-N1 target was specific to the target because ASO had no effect on splicing of *SMN2* minigene if the ISS-N1 target was mutated. At the same time, compensatory mutations within the ASO fully rescued its stimulatory effect in a *SMN2* minigene with the mutated target [58]. As a consequence of these promising results, ISS-N1 became the frontrunner target for developing an ASO-based therapy of SMA. However, new questions pertaining to the role of ASO chemistry, in vivo efficacy and independent validations emerged. With the passing of time, an increasing number of investigators contributed to testing the efficacy of ISS-N1-targeting ASOs using various chemistries and different mouse models of SMA [64]. Findings of these studies had a transformative effect on the field (of SMA and antisense technology for aberrant pre-mRNA splicing correction) as several of the ISS-N1 targeting ASOs drastically pushed the boundaries of therapeutic efficacies known until today.

At the time when ISS-N1 was discovered at University of Massachusetts Medical School (Worcester, MA, USA) [58], ISIS Pharmaceuticals (Carlsbad, CA, USA) was looking to develop an ASO-based drug for SMA. In collaborations with the Krainer laboratory at Cold Spring Harbor Laboratory (New York, USA), ISIS Pharmaceuticals designed and screened multiple ASOs against exonic and intronic targets [82,83]. All ASOs used in these studies carried phosphorothioate backbone and 2'-O-methoxyethyl (MOE) modifications. Of note, ISIS Pharmaceuticals specializes in MOE chemistry and has produced a FDA-approved drug based on MOE chemistry [84]. The most effective ASO turned out to be ASO-10-27 that targets ISS-N1 [83]. ISIS Pharmaceuticals has procured exclusive rights to the ISS-N1 target to develop an antisense drug for SMA. The lead ASO (ASO-10-27) has been branded as ISIS-SMNR_x, and is currently undergoing phase 3 clinical trial [65]. A preclinical study with ASO-10-27 showed unprecedented therapeutic efficacy in a severe model of SMA [85]. In particular, systemic treatment with two high doses (160 mg/kg bodyweight) of ASO-10-27 increased median survival of severe SMA mice from 10 days to 248 days. However, the median survival of severe SMA mice was substantially lower when ASO delivery (20 mg/kg bodyweight) was performed through intra-cerebroventricular (ICV) route. Given the charged backbone of MOE ASOs, it is likely that these molecules (MOE ASOs) cause toxicity in brain. Consistently, authors did not test the efficacy of higher doses (than 20 mg/kg bodyweight) of ASO-10-27 through ICV delivery. Nonetheless, efficacy of systemic administration of ASO-10-27 demonstrated for the first time that the life expectancy of severe SMA model could be extended more than ten-fold, surpassing all previous records of therapeutic efficacy of compounds hitherto tested.

Recent studies with ISS-N1-targeting morpholinos have also shown very promising results [86–89]. Unlike MOE ASOs, morpholino ASOs contain neutral backbone, hence, are likely to exert less adverse effects at high dosages upon ICV administration. Indeed, a single ICV administration of a 20-mer morpholino ASO increased median survival of severe SMA mice

from 14 days to more than 100 days (86). A subsequent study employing a single ICV administration of a 25-mer morpholino ASO (40 mg/kg body weight) increased lifespan of severe SMA mice to an unprecedented 30-fold (87). These results underscore the importance of ASO chemistry and size as critical factors in determining the efficacy of an ASO-based therapy of SMA. Findings of these in vivo studies further pushed the boundaries of therapeutic efficacy of compounds reported thus far for the treatment of SMA. A better efficacy with a single low dose (40 mg/kg body weight) of morpholino ASO compared to two high doses (160 mg/kg body weight) of MOE ASO undoubtedly puts morpholino chemistry as front runner for developing a future drug for SMA therapy.

The results of these initial studies on ISS-N1 provide important insights into the mechanism by which an ISS-N1-targeting ASO promotes exon 7 inclusion. For instance, the negative effect of ISS-N1 was partly retained in a heterologous context [58], suggesting that ISS-N1 is a portable cis-element with a potential to recruit inhibitory factors and/or separate the positive cis-elements away from the 5' ss of an exon. An independent study that confirmed the inhibitory nature of ISS-N1 suggested the role of two hnRNP A1/A2 motifs in conferring the negative effect of ISS-N1 [83]. Of note, the first residue of ISS-N1 is a cytosine that occupies the 10th intronic position (+10C) and does not belong to either hnRNP A1/A2 motifs reported in ISS-N1. Interestingly, sequestration of +10C was found to be absolutely critical for the stimulatory effect of an ISS-N1-targeting ASO [90]. In a subsequent study, it was proposed that ISS-N1 acts as a barrier between the 5' ss of exon 7 and the binding site of stimulatory factors TIA1/TIAR [91]. Hence, any mechanism by which an ASO targeting ISS-N1 produces a stimulatory effect on *SMN2* exon 7 splicing must account for the roles of +10C and TIA1/TIAR that precede and follow the hnRNP A1/A2 motifs within ISS-N1, respectively. The fact that the inhibitory effect of ISS-N1 in a heterologous context is substantially lower than in the wild type context [58], suggests the role of additional inhibitory factors (proteins and RNA sequences/structures) that coordinate with ISS-N1 in the wild type context. Indeed, there are at least three inhibitory cis-elements in the vicinity of ISS-N1. The first among these is TSL2, a terminal stem-loop structure that sequesters the 5' ss of exon 7 (Figures 1 and 2) [63]. The second negative element is a GC-rich sequence (GCRS) that partially overlaps ISS-N1 [59]. The third is an internal stem formed by a long-distance interaction (ISTL1) in which ISS-N2, a deep intronic sequence, interacts with the sequences at the beginning of intron 7 (Figures 1 and 2) [60]. Hence, an ISS-N1-targeting ASO is likely to trigger several interconnected events including displacement of hnRNP A1/A2, abrogation of ISTL1 and recruitment of TIA1/TIAR (Figures 2 and 3) [93]. As a result, the 5' ss of exon 7 becomes more conducive for the recruitment of U1 snRNP that plays a critical role in setting the stage for intron 7 removal - (Figure 3).

As mentioned earlier, other ASO targets used for correction of *SMN2* exon 7 splicing include GCRS, ISS-N2 and element 1. The GCRS is an 8-nt long sequence and partially overlaps with ISS-N1 [59]. This is the sole GC-rich sequence in the first half of intron 7. Sequestration of the GCRS by an 8-mer 2OMe ASO (3UP8) fully restored *SMN2* exon 7 inclusion in SMA patient cells [59]. There is a parallel between mechanisms of ISS-N1- and GCRS-targeting ASOs. In both cases, ISTL1 is abrogated due to sequestration of 10C

(Figure 2). This in turn brings structural changes conducive for the recruitment of U1 snRNP at the 5' ss of exon 7 (Figure 3). An improved version of 3UP8 (3UP8i) with terminal modifications showed promising results in two mouse models of SMA (94). Unlike ISS-N1 and the GCRS, ISS-N2 is located away from the 5' ss of exon 7 and yet communicates with the 5' ss through ISTL1 (Figures 1B and 3) [60]. A 15-mer ISS-N2-targeting 2OMe ASO (ASO 283–297) stimulated *SMN2* exon 7 inclusion in SMA patient cells [60]. The mechanism by which the ISS-N2-targeting ASO promotes *SMN2* exon 7 inclusion involves the disruption of ISTL1 and possibly other structural changes conducive for the recruitment of U1 snRNP at the 5' ss of exon 7 [93]. Element 1 is a poorly characterized long inhibitory element within intron 6. The first study with a 16-mer element 1-targeting 2OMe ASO (oligo-element1) produced a moderate stimulatory effect on exon 7 splicing in the context of a minigene [57]. Recently, a 26-mer element 1-targeting morpholino ASO (E1^{MO}-ASO) has shown promising results in a mouse model of SMA [95]. The mechanism by which element 1-targeting ASOs stimulate *SMN2* exon 7 inclusion may involve displacement of negative factors leading to the remodeling of the 3' ss of exon 7.

Antisense therapy based on splice site suppression

Considering that skipping of *SMN2* exon 7 is caused by pairing of the 5' ss of exon 6 with the 3' ss of exon 8, sequestration of the 3' ss of exon 8 will likely prevent or delay this pairing. In addition, sequestration of the 3' ss of exon 8 may force the inclusion of exon 7 by pairing of the 5' ss of exon 6 with the 3' ss of exon 7. In this case intron 7 will be retained due to unavailability of the 3' ss of exon 8. Considering exon 7 is the last coding exon, *SMN2* mRNA transcript with retained intron 7 will theoretically be able to make the full length SMN. However, this outcome is conditional: the intron 7-containing mRNA should be efficiently exported from the nucleus. Moreover, the translation of this transcript, which now has the newly-acquired long 3' untranslated region (3'UTR), should not be blocked by microRNAs. Of note, a transcript with novel 3'UTR may perturb the expression of several proteins by titrating away microRNAs from their corresponding mRNAs [96]. An early study employed 2OMe ASO (Oligo_{NCT}) to block the 3' ss of exon 8 and tested the effect on exon 7 splicing [56]. While Oligo_{NCT} did not completely prevent exon 7 skipping, it did increase the levels of exon 7 included transcripts with or without intron 7 retention. The fact that the removal of intron 7 is not mechanistically possible if the 3' ss of exon 8 is sequestered, it is likely that Oligo_{NCT} promoted intron 7 removal by binding to other targets. A subsequent study used a vector-based expression of ASOs sequestering the 3' ss of exon 8 [97]. Several ASOs that sequestered the junction of intron 7 and exon 8 showed a reduction in the levels of *SMN2* exon 7 skipped transcripts accompanied by an increase in the levels of SMN in HeLa cells. A similar approach produced increased levels of full-length *SMN2* transcripts in SMA patient cells in which an ASO targeting the 3' ss of *SMN2* exon 8 was expressed in the context of U7 snRNP, which protects against hydrolysis by ribonucleases [98]. However, since intron 7-retained products were not detected, it is likely that the ASO is working through additional mechanisms other than the expected sequestration of the 3' ss of *SMN2* exon 8.

Bifunctional ASOs for SMA therapy

A bifunctional ASO is comprised of two distinct sequence motifs i.e. antisense and tail motifs. The antisense motif is the complementary sequence against a predetermined target site; whereas, the tail motif is a hanging sequence that interacts with and/or recruits splicing factor(s). Early studies on bifunctional ASOs conducted more than a decade ago were aimed at delivering positive splicing factors to the 3' ss of exon 7 [71,72]. A recent more systematic study compared the efficacy of multiple bifunctional ASOs targeting *SMN2* exon 7 in vitro as well as in patient cells [99]. This study also examined the impact of oligonucleotide modifications on the efficacy of bifunctional ASOs. A complex pattern emerged with respect to the efficacy of these ASOs. A 38-nt bifunctional ASO, oligonucleotide 1, carrying a 23-nt unmodified tail and a 15-nt 2OMe antisense motif that annealed from the 2nd to 16th positions of exon 7 turned out to be the most effective bifunctional ASO based on both in vitro and cell-based assays [99]. Oligonucleotide 1 retained its stimulatory effect even when the annealing position was shifted to exon 6. The 23-nt unmodified tail in oligonucleotide 1 is comprised of three AGGAGG motifs separated by C residues. Notably, modifications of the sugar-phosphate backbone in the tail motif of oligonucleotide 1 were less effective. This could be due to perturbations in the interface of RNA-protein interactions. The Authors hypothesized that the stimulatory effect of oligonucleotide 1 is due to interaction of the tail sequence with Tra2 β and Tra2 β -interacting proteins. A more recent study showed that the tail motif of oligonucleotide 1 interacts with several proteins; some of these interactions result in various types of nonproductive complexes [100]. This explains why a very high concentration (250 nM) of oligonucleotide 1 is required to observe the maximum efficacy [99]. As a substitute to the delivery of high concentrations of bifunctional ASOs, continuous expression of exon 7-targeting bifunctional ASOs employing vector-based approaches has been examined [101–103]. Significantly, expression of an U7 snRNP-derived bifunctional ASO targeting *SMN2* exon 7 produced ~20-fold increase in the life expectancy of a severe mouse model of SMA and improved neuromuscular junction properties [102,103]. This suggests that high concentrations of transacting factors, including components of U7 snRNP in the vicinity of exon 7, have the potential to modulate disease severity. Bifunctional ASOs targeting the 3' ss of *SMN2* exon 8 [104,105], element 1 within *SMN2* intron 6 [106] and ISS-N1 within *SMN2* intron 7 have also been employed [107]. However, the results of these experiments were somewhat less impressive due to relatively low efficacy in vivo. One of the disadvantages of bifunctional ASOs is the permanent entrapment of one or more transacting factor(s). This may produce unintended negative consequences on cellular metabolism, as entrapped factors may not be available for their designated functions. Further, due to its large size, a bifunctional ASO may be more likely to produce off-target effects through annealing to the partially complementary sequences. Supporting this argument, none of the bifunctional ASOs reported thus far have an efficacy comparable to that of the MOE or morpholino ASO that targets ISS-N1.

Dual-masking ASOs for SMA therapy

Dual-masking and double targeting refers to simultaneous sequestration of two distinct targets within a pre-mRNA. However, unlike double targeting in which two different ASOs

simultaneously target two distinct sites, dual-masking employs a single ASO molecule to simultaneously target two distinct sites. Studies with double targeting 2OMe ASOs and U7 snRNP-associated ASOs did not provide any significant benefits in splicing correction [61,97]. Recently, several dual-masking 2OMe ASOs have been evaluated by targeting two of the four known inhibitory cis-elements [61]. These cis-elements are: element 1, ISS-N1, +100G (the 100th position of intron 7) and the 3' ss of exon 8 (Figure 1A). The most promising result was obtained by Dual-1, a 39-nt ASO that simultaneously sequestered ISS-N1 and the 3' ss of exon 8. The annealing position of Dual-1 spanned from 9th to 29th positions of intron 7 and from -13th to +5th position of exon 8 (Figure 4). Similar to an ISS-N1-targeting ASO, Dual-1 is predicted to displace hnRNP A1/A2, abrogate ISTL1, and recruit TIA1/TIAR at the 5' ss of exon 7. In addition, Dual-1 brings the 5' ss and 3' ss of intron 7 in close proximity (Figure 4). Such an outcome would favor an intron definition model as a mechanism to promote *SMN2* exon 7 inclusion [69]. Consistent with the strong stimulatory effect on *SMN2* exon 7 splicing, Dual-1 substantially elevated the levels of SMN in SMA patient cells [61]. The results were more promising at the highest tested concentration of 200 nM. Unlike bifunctional ASOs, dual-masking ASOs do not require a transacting factor for their function. The efficacy of Dual-1 appears to exceed all chemically synthesized bifunctional ASOs reported thus far. However, it remains to be determined if a dual-masking ASO retains its efficacy in vivo.

Future perspective

Approaches to correct *SMN2* exon 7 splicing employing ASO-based strategies have come a long way. While early in vitro and cell-based studies were aimed at testing the proof of principles, subsequent in vivo studies have furnished promising results with great significance to therapy. A series of seminal discoveries employing the ISS-N1 target provides a roadmap for future pre-clinical studies with other ASOs. The most valuable and rather fortunate lesson learnt from the ISS-N1 target is the reproducibility of results in different laboratories in different mouse models employing different oligonucleotide chemistries. Unfortunately, it is not always the case that major claims of one laboratory are reproducible in another laboratory [108–110]. The healthy tradition of independent validation is key to propel discoveries from basic laboratory studies to clinical trials. As the list of therapeutic compounds for the treatment of SMA grows, the need for independent validation has never been greater. This is particularly true for proprietary compounds that are not commercially accessible and/or remain very expensive to synthesize. In any scenario, there are hurdles in realizing acceptable clinical outcomes based on the otherwise promising results of cell- and/or animal-based studies. A key fact to reconcile is that not all ASO-based approaches are analogous even when the same target is used. For instance, a single dose of a synthetic ASO will likely produce a different outcome than the continuous expression of the same ASO employing a vector. In many respects, vector-based delivery of an ASO is analogous to gene therapy that promises full-length SMN by continuous supply of transcripts from the exogenously delivered gene. Hence, it is unlikely that a vector-based delivery of an ASO will translate into therapy until the advantages of this approach over gene therapy are clearly demonstrated. Nonetheless, vector-based delivery remains an important tool to assess the impact of the sustained expression of an oligonucleotide or a

transacting factor on disease severity in a mouse model of SMA. These same considerations for SMA therapy development also apply to any other disease.

One of the attractive aspects of therapy based on a chemically synthesized ASO is the potential for replacement of today's working ASO with tomorrow's improved ASO as the technology becomes advanced. While success of ISIS-SMNR_x will not be known for sometime, there is a need for additional targets so that other oligonucleotides and chemistries could be employed for SMA therapy. There is no universal rule with respect to the size of an ASO for in vivo application. Large and small ASOs carry their own advantages and disadvantages. A large ASO has disadvantages of: (i) self sequestering by folding into secondary and high-order structures, (ii) forming non-productive complexes by interacting with cellular proteins, and (iii) producing off-target effects due to high tolerance for mismatch base pairing. A small ASO has disadvantages of: (i) being diluted by interactions with multiple probable targets in the genome/transcriptome, and (ii) having fast clearance in the blood. However, there are several advantages unique to a small ASO. Most important among these are the low cost of synthesis, low tolerance for mismatch base pairing and easier transport across biological barriers. The low tolerance for the mismatch base pairs results into the low off-target effects and a high specificity for the target. Furthermore, a small ASO offers feasibility of synthesis of a large number of variants with different modifications at every single position of the oligonucleotide. Of note, the potential of ASO-based therapy remains grossly underutilized due to the lack of studies on optimization of modifications at every position of an oligonucleotide. An antisense compound should be treated no different than a small molecule in which a single modification could bring desirable features such as improved stability, high specificity and reduced toxicity. Indeed, terminal modifications of the 8-mer-2OMe ASO targeting the GCRS turned out to be critical for in vivo efficacy [94]. One of the major hurdles of an ASO-based therapy for neurodegenerative diseases is the inability of an ASO to cross blood brain barrier. Any breakthrough in this direction would have a transformative impact for the treatment of these diseases.

Success of an ASO-based therapy in SMA has implications for the treatment of other genetic diseases requiring a splicing correction. Progress thus far has been very bright and the future of an ASO-based therapy for SMA looks even brighter. An ASO-based approach described here will potentially benefit all sub-types of SMA patients affected by the loss of *SMN1*. For a wide application of an ASO-based approach, modalities of time, dose and method of delivery will have to be worked for different patient groups. A combination therapy with ASO-based approach as one of the main ingredients of the treatment may provide a better outcome. However, compatibility of other ingredient(s) in combination therapy remains a matter of future investigation.

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discovered in the Singh lab at UMASS Medical School (MA, USA). Inventors, including RNS, NNS and UMASS Medical School, are currently benefiting from licensing of ISS-N1 target to ISIS Pharmaceuticals. Iowa State University holds intellectual property rights on GCRS and ISS-N2 targets. Therefore, inventors including RNS, NNS and Iowa State University could potentially benefit from any future commercial exploitation of GCRS and ISS-N2 targets.

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Key Terms

Key terms for “Genetics of Spinal muscular atrophy”

Spinal muscular atrophy Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality.

SMN SMN is a multifunctional protein with distinct functional domains including tudor, nucleic acid binding, self-association, YG-box, proline-rich and calpain cleavage domains.

Key terms for “Therapeutic approaches of SMA”

Antisense oligonucleotide Antisense oligonucleotides (ASOs) are nucleic acid molecules that anneal to specific RNA or DNA sequences. ASOs have been used to block regulatory sequences to modulate alternative pre-mRNA splicing.

ISS-N1 ISS-N1 is a 15-nt long intronic splicing silencer located from the 10th to 24th position of *SMN* intron 7. ISS-N1 is the leading target for an ASO-mediated splicing correction in SMA.

ISIS-SMN_{Rx} ISIS-SMN_{Rx} is an ASO-based drug currently undergoing phase 3 clinical trial for the treatment of SMA. Drug is based on proprietary MOE chemistry of ISIS Pharmaceuticals and targets ISS-N1 sequence within *SMN2* intron 7.

Key terms for “Antisense therapy based on canonical targets”

GCRS GCRS is an 8-nt long intronic splicing silencer located from the 7th to 14th position of *SMN* intron 7. GCRS is the smallest validated target for an ASO-mediated splicing correction in SMA.

ISS-N2 ISS-N2 is a 23-nt long deep intronic sequence located from the 275th to 297th position of *SMN* intron 7. ISS-N2 is a recently validated target for an ASO-mediated splicing correction in SMA.

Element 1 Element 1 is a long inhibitory cis-element within *SMN* intron 6.

Key terms for “Antisense therapy based on splice site suppression”

U7 snRNP derived ASOs U7 snRNP is a RNA-protein complex involved in the 3' end maturation of histone pre-mRNAs. The RNA component of U7 snRNP could be modified to include antisense sequence for splicing modulation. Targeting of exon 7 or the 3' ss of exon 8 by ASOs expressed in the context of the U7 snRNP promote *SMN2* exon 7 inclusion.

Key terms for “bifunctional ASOs for SMA therapy”

Bifunctional ASO A bifunctional ASO is comprised of two distinct sequence motifs i.e. antisense and tail motifs. The antisense motif anneals to a

predetermined target site within *SMN2* pre-mRNA and the tail motif recruits splicing factor(s) for stimulation of *SMN2* exon 7 inclusion.

Key terms for “Dual-masking ASOs for SMA therapy”

Dual-masking ASO	A dual-masking ASO simultaneously sequesters two distinct sites within <i>SMN2</i> pre-mRNA.
Dual-1	Dual-1 is a 39-nt long ASO that simultaneously sequestered ISS-N1 and the 3' ss of exon 8.

Key terms for “Future perspective”

Independent validation	Independent validation is a critical to propel discoveries from basic laboratories to clinical trials.
Position-specific optimization of an oligonucleotide	Optimization of modifications at every position of an oligonucleotide is key to improve the efficacy of a therapeutic ASO.

Executive summary

Genetics of spinal muscular atrophy

- Low levels of the multifunctional SMN protein primarily due to deletion or mutations of *SMN1* cause SMA, the leading genetic cause of infant death.
- *SMN2*, a nearly identical copy of *SMN1*, cannot fully compensate for the *SMN1* loss due to predominant skipping of *SMN2* exon 7, leading to the production of SMN 7, a truncated protein that is rapidly degraded.
- Defects caused by the lack of *SMN1* can be partially compensated by the presence of *SMN2*, which produces low levels of full-length SMN.
- Severity of SMA is likely to depend upon several factors that regulate SMN levels through transcription, splicing, translation, posttranslational modifications and protein-protein interactions.
- Factors independent of SMN could also affect the severity of SMA and/or cause SMA.

ASO-based therapy of SMA

- A canonical ASO-based therapy of SMA is based on promoting *SMN2* exon 7 inclusion by an oligonucleotide that binds to a specific sequence (target) within *SMN2* pre-mRNA.
- Potential targets of an ASO-based therapy of SMA include the 3' ss of exon 8, element 1, ISS-N1, GCRS, ISTL1, ISS-N2 and the 100th position of *SMN2* intron 7.
- Stimulatory effect of an ASO could be due to displacement of a negative regulator, recruitment of a positive regulator and formation of a favorable RNA structure.
- ASOs annealing to sequences away from the *SMN2* specific mutations (C6U and A+100G) could have dramatic effects on exon 7 splicing.

Highlights of ISS-N1 target under clinical trial

- Located close to the 5' ss of exon 7, the 15-nt long ISS-N1 is a strong negative regulator of *SMN2* exon 7 splicing.
- ISS-N1 harbors two putative binding sites for inhibitory factor hnRNP A1/A2.
- The first position of ISS-N1 is a cytosine residue that participates in a unique RNA structure formed by a long-distance interaction.
- ISS-N1 suppresses accessibility of the 5' ss of exon 7.
- ISS-N1 is the leading target for an ASO-based therapy of SMA.
- A series of independent studies carried in different mouse models of SMA validates the therapeutic efficacy of ISS-N1-targeting ASOs

- ISIS-SMNR_x, an ISS-N1-targeting drug, is currently undergoing phase 3 clinical trial. If successful, ISIS-SMNR_x will become the first ASO-based drug to elevate the levels of a fully functional protein by stimulating the inclusion of an exon.

GCRS target for splicing correction by a small ASO

- The 8-nt long GCRS is a negative regulator of *SMN2* exon 7 splicing.
- GCRS is the only known target for an ASO-mediated splicing correction by a small ASO.
- Cell-based and in vivo studies validate the therapeutic efficacy of an 8-mer ASO targeting GCRS.

Bifunctional ASOs for splicing correction in SMA

- A bifunctional ASO anneals to a specific location within *SMN2* pre-mRNA and recruits splicing factor(s) to promote exon 7 inclusion.
- A vector-based expression of a U7 snRNP containing bifunctional ASO has been found to dramatically reduce the phenotype severity of a mouse model of SMA.

Dual-masking ASOs for splicing correction in SMA

- A single molecule of a dual-masking ASO simultaneously sequesters two negative cis-elements on *SMN2* pre-mRNA to promote exon 7 inclusion.
- Simultaneous sequestration of ISS-N1 and the 3' ss of exon 8 by a dual-masking ASO produced a better splicing correction efficacy than an ASO that targets ISS-N1 alone.

Future perspective

- Availability of other novel targets offer the potential for developing additional ASO-based therapies for SMA.
- There is a need to exploit the benefits of modification of every single position of an ASO.
- Development of an ASO capable of crossing blood brain barrier would have a transformative impact for the treatment of neurodegenerative diseases.

Figure 1. Targets of SMA therapy

(A) Relative position of major targets for an ASO-mediated splicing correction in SMA.

Neutral and positive numbering start from the first positions of exon 7 and intron 7, respectively. Negative numbering starts from the last position of intron 6. Element 1 occupies positions from -68 to -112 in intron 6 [57]. ISS-N1 and GCRS occupy positions from +10 to +24 [58] and from +7 to +14 [59] in intron 7, respectively. 5'ISTL1 and 3'ISTL1 refer to the 5' and 3' strands of ISTL1, respectively [60]. An ASO-based approach has been used to target the 100th position of intron 7 [61]. A+100G refers to an *SMN2* specific mutation at the 100th position of intron 7 [62]. TSL2 is an inhibitory stem-loop structure that sequesters the 5' ss of exon 7 [63]. (B) Relative location of cis-elements in the context of the RNA secondary structure of exon 7 and intron 7. Numbering is the same as in

panel A. Presented structures of exon 7 and intron 7 were deduced from enzymatic and chemical structure probing [60,63].

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Figure 2. Effect of ISS-N1 and GCRS-targeting ASOs on accessibility of the 5' splice site of exon 7
 The *SMN2* mRNA is numbered from the start of exon 7 (neutral numbers) and the start of intron 7 (positive numbers) with a rainbow scheme ranging from blue to red in the 5' to 3' direction for the cartoon depiction of the phosphate backbone in the 3D models. Nucleotide bases in all figures are colored by type: green for adenine, blue for cytosine, orange for guanine and red for uracil. Secondary structures include base pairing used for model prediction indicated by a solid dash and additional base pairs formed during model calculations denoted by a tilde. All model calculations included a construct of the 5' splice site of

exon 7 comprising 100 nucleotides ranging from 33rd position in exon 7 to +300th position in intron 7 with a 225 nucleotide deletion between +57th and +283rd replaced by a three nucleotides to complete a UAAC tetraloop. A hierarchical stepwise assembly protocol within the Rosetta modeling suit (version 2015.09.57646) was used to build local secondary structure motifs and assemble the motifs into a tertiary model that was further refined by energy minimization [92]. **(A)** In the absence of ASO binding, the 3D model of the 5' ss of exon 7 (shown on the right) was calculated with experimental base pairing restraints derived from SHAPE-based chemical probing of the secondary structure (shown on the left). The model prediction includes two stem-loop hairpin motifs, TSL2 (38th position in exon 7 to +2nd position in intron 7) and TSL3 (+17th to +41st positions); and two long-range stem interactions, ISTL1 (+3rd to +10th and +290th to +297th positions) and ISTL2 (+50th to +56th and +283rd to +289th positions). **(B)** Predicted changes in the three dimensional structure of the 5' ss of exon 7 upon binding of an ISS-N1 targeting 18-mer ASO (F18) (shown on the right) include disruption of the TSL3 motif and ISTL1 structure. Annealing position of F18 is marked on the secondary structure (shown left). **(C)** The three dimensional structure of the 5' ss of exon 7 upon binding of an ISS-N1 targeting 8-mer ASO (F8) (shown right) predicts retention of all stem-loop motifs and long-range stem interactions. Annealing position of F8 is marked on the secondary structure (shown left). **(D)** Predicted changes in the three dimensional structure of the 5' ss of exon 7 upon binding of a GCRS targeting 8-mer ASO (3UP8) (shown right) include disruption of the ISTL1 structure. Annealing position of 3UP8 is marked on the secondary structure (shown left).

Figure 3. Mechanism of SMN2 exon 7 splicing correction by an ISS-N1-targeting ASO
Proposed mechanism by which an ISS-N1-targeting ASO promotes exon 7 inclusion. An ISS-N1 targeting ASO sequesters binding sites of hnRNP A1/A2, disrupts/destabilizes TSL3, ISTL1 and ISTL2. This leads to binding of TIA1 to now accessible sites (shown in green) and enhanced recruitment of U1 snRNP to the 5' ss of exon 7. Mechanism is adapted from [60]. Numbering is the same as in Figure 1A.

Figure 4. Effect of a dual-masking ASO on accessibility of the 5' ss of exon 7
Annealing of a dual-masking ASO (Dual-1) to ISS-N1 and the 3' ss of exon 8 brings the 5' ss of exon 7 and 3' ss of exon 8 in close proximity. Dual-1 is described in [61]. Remaining structure of intron 7 is shown. Numbering is the same as in Figure 1A.